OSMOTIC PROPERTIES OF THE ERYTHROCYTE

III. The Applicability of Osmotic Laws to the Rate of Hemolysis in Hypotonic Solutions of Non-electrolytes

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I

Few subjects on the field of cellular physiology have received as much attention as that of osmotic hemolysis. Beginning with the observations of Hamburger (1886)—for a summary of the early literature, see Hamburger (1902)—there have appeared literally hundreds of papers dealing with this process under all conceivable conditions of health and disease, and of age, sex, species, and previous treatment of the blood. As has been pointed out in the preceding paper of this series (Jacobs and Parpart, 1931), much of this work is of very doubtful value because of the neglect of certain essential experimental precautions. Entirely apart from this defect, however, the work is disappointing in its almost complete failure to deal with the question of the rate at which such hemolytic processes occur. Unlike the published work upon other types of hemolysis (saponin, bile salts, specific sera, etc.), that upon osmotic hemolysis with few exceptions has had to do merely with the end state finally reached by the system. Information about this point, though possessing a limited practical value in medicine, is, on the whole, of comparatively little theoretical interest. So-called "fragility" studies—at least as ordinarily carried out—are concerned with the specific properties of the erythrocyte alone and they throw little light upon more fundamental problems of cell physiology. On the other hand, a study of the *rate* of osmotic hemolysis, which is closely associated with the rate of entrance of water into the cell, has obvious applications to many important general problems in the fields of osmosis, cell permeability, etc.

To all investigators who have tried in the usual way to measure the rate of osmotic hemolysis the reason for the almost complete neglect of this field in the past is clear. Osmotic hemolysis, when it goes to completion, as, for example, in distilled water or in very strongly hypotonic solutions, is such a rapid process—requiring for its entire course perhaps only a few seconds—that the ordinary methods of

studying hemolytic phenomena cannot be successfully employed with it. On the other hand, when it is sufficiently slow to permit ready measurement, especially when the end-point is some partial degree of hemolysis, the results obtained are likely to be so variable and irregular, and at first sight so generally inexplicable, that most persons who have tried to work under these conditions have soon abandoned their attempts. The author has pointed out elsewhere (Jacobs, 1927, 1928, 1931; Jacobs and Parpart, 1931) that while irregularities in the behavior of the erythrocyte can be minimized by a strict standardization of the experimental procedure, they are in part inherent in the nature of the material itself and are therefore unavoidable. The difficulty, in brief, is that factors such as temperature, pH, etc. which might be expected to affect the rate at which a given equilibrium condition is attained, have an unusually strong tendency in the erythrocyte to change at the same time the position of the equilibrium itself. Under these conditions the results are, in general, too complicated for ready analysis; and the experimenter is of necessity driven back to the other horn of the dilemma where the difficulty is with the rapidity of the process and therefore with the method rather than with the material.

Fortunately, the simple method of the author (Jacobs, 1930) for the study of hemolysis proves to be adequate for a fairly accurate determination of times of hemolysis greater than approximately 1.2 seconds and permits experiments on the rate of the process to be made under conditions where disturbing equilibrium factors are of negligible importance, namely, in distilled water and in very strongly hypotonic solutions. The method therefore opens to experimental study an important field which has heretofore been almost wholly neglected. This general field will be dealt with in the present and in several succeeding papers.

A very fundamental question, which must first be decided before other work can be undertaken with profit, is how far the rate of hemolysis in hypotonic solutions may be considered to depend upon the rate of entrance of water into the erythrocyte in accordance with simple osmotic laws. In an earlier paper by the author (Jacobs, 1927) it was tentatively assumed that the erythrocyte behaves as a simple osmometer and gives up its hemoglobin to the surrounding solution when a certain critical hemolytic volume, V_h, is reached. On this assumption equations were derived for the calculation of permeability constants for water from data on the rate of osmotic hemolysis. It was, however, emphasized in another place (Jacobs, 1931) that osmotic hemolysis is in reality a fairly complicated process involving (*a*) the entrance into

the cell of water, (*b*) the escape of hemoglobin, (*c*) the possible escape of salts and other osmotically active materials and (*d*) changes produced in various other ways in what is commonly loosely spoken of as the osmotic resistance of the cell. Only where factors *b*, *c* and *d* can be shown to be of negligible importance is it permissible to use the simple method of treating the subject previously employed; and a more critical examination of this point is therefore highly desirable.

A further need for such an examination is created by the recent work of Ponder and Saslow (1931), who have given reasons for doubting the applicability of simple osmotic laws to the erythrocyte because of the leakage from the cell during the course of at least certain types of experiments of osmotically active materials (factor *c* mentioned above). It must be admitted that in cases where such leakage occurs to any considerable extent a simple treatment of the problem is impossible. However, in view of the fact that Ponder and Saslow dealt primarily with final equilibria, arrived at in the course of a considerable time, it is by no means certain that in hemolytic experiments whose duration is only a few seconds such leakage as they have described would be a disturbing factor, though it is not impossible that it might. The question can be settled only by experiment, preferably by a comparison of the observed rates of osmotic hemolysis with those deduced according to the theory that the erythrocyte behaves as an ideal osmometer. Such a comparison will now be made.

II

The equations given in the earlier paper (Jacobs, 1927) for relating the time of hemolysis to the concentration of the medium are somewhat inconvenient because they employ the initial volume, V_0, and the hemolytic volume, V_h, of the cell. Though it has recently been shown by Ponder and Saslow (1931) that the idea of a hemolytic volume has actual experimental justification, there is, in general, a certain ambiguity in working with volumes. This is due to the fact that the volume that enters into osmotic equations is not the measured volume of the cell but rather that of the water which the cell contains. In some cases, *e.g.*, the cells of the plant *Tradescantia* (Höfler, 1917), this distinction is unimportant, but in the erythrocyte, which is loaded with hemoglobin to an extent of over 30 per cent by weight, it undoubtedly is—though, unfortunately, there is little agreement among different workers as to the actual magnitude of the true volume. For many purposes it is perhaps best to use the weight of the water in the cell as determined by chemical analysis (Van Slyke, Wu and McLean, 1923, etc.), but since under ordinary experimental conditions the

weight of the water and what might be called the osmotically effective volume of the cell, *i.e.*, that part of the total volume which takes part in osmotic changes, are approximately related in a very simple manner, it is perhaps permissible for more ready comparison with other published work in this field to retain for the present purposes the older type of osmotic equations involving volumes and concentrations.

Assuming with Hill (1930) that the water within the cell is almost entirely "free," *i.e.*, capable of taking part in osmotic equilibria, or at least that there are no marked changes during the course of the experiment in the degree of "binding" of water by cell constituents, we have the relation:

$$cV = c_0 V_0,$$

where c_0 and V_0 are the initial osmolar concentration and osmotically effective volume of the cell, respectively, and c and V are any other corresponding pair of these variables. The hemolytic volume, V_h, used in previous discussions may therefore be expressed in terms of constants and of the more convenient hemolytic concentration:

$$V_h = V_0 \frac{c_0}{c_h}.$$

Making certain necessary and probably well justified simplifying assumptions as to the nature of the diffusion of water across the membrane of the erythrocyte (see in this connection Northrop, 1927; Lucké, Hartline and McCutcheon, 1931; Jacobs and Stewart, 1932), it may be predicted that the rate at which it will enter the cell, *i.e.*, the rate of increase of the cell volume, will at any given instant be proportional to the difference in the osmotic pressures, and therefore to that of the concentrations of the internal and external solutions, and to the extent of surface of the cell.

$$\frac{dV}{dt} = kA(c - C). \tag{1}$$

The external concentration, C, may be considered to be constant since the volume of the surrounding solution is very large as compared with that of the suspended cells (approximately 1000 : 1 in these experiments). Since

$$V = V_0 \frac{c_0}{c},$$

we may write equation (1) in the form:

$$-\frac{c_0 V_0}{c^2} \frac{dc}{dt} = kA(c - C). \tag{2}$$

Fortunately in the erythrocyte, because of its peculiar biconcave shape, a considerable degree of increase in volume is possible without any change in surface. No great error will result, therefore, if A be treated as if it were constant, and this simplifying assumption permits equation (2) to be integrated at once after separating the variables; that is:

$$kAt = -c_0 V_0 \int \frac{dc}{c^2(c - C)}.$$

Remembering that when $t = 0$, $c = c_0$, the initial isotonic concentration for blood, we finally obtain:

$$kAt = \frac{c_0 V_0}{C^2} ln \frac{cc_0 - cC}{cc_0 - c_0 C} + \frac{c_0 V_0}{C}\left(\frac{1}{c_0} - \frac{1}{c}\right). \tag{3}$$

For the special case where the cells swell in distilled water, and C in equation (2) is therefore equal to zero, a simpler equation results, namely,

$$kAt = \frac{c_0 V_0}{2}\left(\frac{1}{c^2} - \frac{1}{c_0^2}\right). \tag{4}$$

As has already been stated, V_0, the initial osmotically effective volume of the cell, is not exactly known but is, in any case, a constant. For our present purposes, therefore, V_0 and A, which has also been treated as a constant, may be incorporated with the true permeability constant, k, to give a quantity, k', whose constancy over a range of concentrations would equally well furnish a proof of the correctness of equations (3) and (4). Since we wish to use the equations only for the point at which hemolysis occurs, namely t_h, c_h, we substitute these particular values for t and c, respectively, and also for convenience represent the ratio c_0/c_h by R, giving finally:

$$k't_h = \frac{c_0}{C^2} \ln \frac{c_0 - C}{c_0 - RC} - \frac{R - 1}{C} \tag{5}$$

and

$$k't_h = \frac{1}{2c_0}(R^2 - 1). \tag{6}$$

If, therefore, the erythrocyte behaves as a simple osmometer, equations (5) and (6) should yield the same value of k' for all values of C including the value of zero when distilled water is employed.

III

To test the applicability of the theoretical equations derived in the preceding section to osmotic hemolysis, experiments were performed upon erythrocytes by the method mentioned above (Jacobs, 1930). In the present paper only a single one of the earlier experiments with

saccharose solutions will be described in detail; but it may be mentioned that essentially the same results have been obtained in a considerable number of other experiments both with this substance and with dextrose. Some of the later confirmatory experiments were performed by A. K. Parpart, whose careful assistance is gratefully acknowledged.

The blood used for the experiment here described was that of the ox, defibrinated immediately after its collection. Because of certain abnormalities that seem to develop when erythrocytes stand for some time in contact with protein-free salt solutions (Kerr, 1929), the cells were not "washed" but were kept in the approximately normal surroundings furnished by their own serum up to the instant of exposure to the hemolytic solutions. It should be noted that previous washing in isotonic non-electrolyte solutions is also contraindicated by the tendency shown by erythrocytes to become agglutinated in such

TABLE I

Times of hemolysis of ox erythrocyte in water and in saccharose solutions. R is assumed to have a value of 2.1.

Concentration	Freezing point depression	Observed time of hemolysis in seconds	k'
0.00	0.00	1.40	2.10
0.10	0.0186	1.53	2.02
0.02	0.0373	1.60	2.05
0.04	0.0747	1.65	2.26
0.06	0.1123	1.73	2.49
0.08	0.1501	1.90	2.70
0.10	0.1880	2.15	3.00
0.12	0.2261	2.50	3.55
0.13	0.2452	2.75	4.12
0.14	0.2643	3.53	4.77
0.145	0.2739	7.33	3.88

solutions. Though the procedure that was of necessity followed resulted in the introduction into the non-electrolyte solutions of slight traces of electrolytes and of proteins, these were small since the dilution of the blood employed was approximately 500 : 1 and that of the serum therefore of the order of 1000 : 1, giving a final concentration of electrolytes in the vicinity of M/6000.

With each solution four determinations of the time of hemolysis were made to the nearest tenth of a second. These figures have been averaged to give the times listed in the third column of Table I. As a rule, the individual observations in each group of four varied by only one or two-tenths of a second. Only with the highest concentrations employed, where the complicating factors previously mentioned are

present, did the observations fail to show a high degree of reproducibility. The concentrations of the solutions employed are given in the first column of the table, but for purposes of calculation the freezing point depressions were used as being more nearly proportional to the osmotic pressures than the concentrations. These were calculated by the empirical equation:

$$\Delta = 1.86C - 0.2C^2,$$

which fits very closely the data given in the "International Critical Tables" for saccharose over the range of concentrations employed.

The critical concentration for 75 per cent hemolysis, which is in practice a convenient end-point to use, was directly determined as 0.148M with a calculated freezing point depression of 0.280°. Since the freezing point depression for ox serum is in the vicinity of 0.58° (Hamburger, 1902) R, the ratio of c_0 to c_h may be taken as approximately 2.1.

From these data and from the observed rates of hemolysis, Table I has been prepared. An inspection of the figures in the first and third columns of this table, or, better, of the positions of the solid circles in Fig. 1, shows the relation between the observed times of hemolysis and the concentrations of the external solutions. It will be noted that an increase in concentration from zero (distilled water) to 0.12M or 0.13M has only a relatively slight retarding effect. The retardation then increases at a rapid rate and at a concentration of 0.148M reaches infinity. This type of curve, characterized by the relative suddenness of its final rise, has always been obtained with non-electrolytes, but, as will be shown in a later paper, not with electrolytes. How far does it agree with the theoretical predictions made by the use of equations (5) and (6)?

The answer to this question may be presented in two ways. In the first place, in the last column of Table I are found the calculated values of k' for the various concentrations employed. It will be noted that while there is good agreement between the values for water and for 0.01M and 0.02M saccharose, this agreement soon disappears and the last values of k' are about 100 per cent greater than the first ones. This lack of agreement is almost certainly not the result of fortuitous errors of observation, since the drift from smaller to larger values of k' is a very regular one. Evidently the predictions made from the theoretical equations depart rather widely from the observed data.

The same thing is shown still more clearly in Fig. 1 where the curve labeled 2.1 represents the times at which hemolysis ought theoretically to occur if the first few values of k' applied throughout the concen-

tration range instead of increasing as they do with increasing concentration. It will again be noted that the agreement between theory and observation is very poor. More specifically, the observed values increase much too slowly until the highest concentrations are reached, and then they tend to increase very rapidly. It would appear, therefore, either that the rate of hemolysis is not governed in any very simple manner by osmotic laws or that some additional factor of importance has been overlooked in making the calculations.

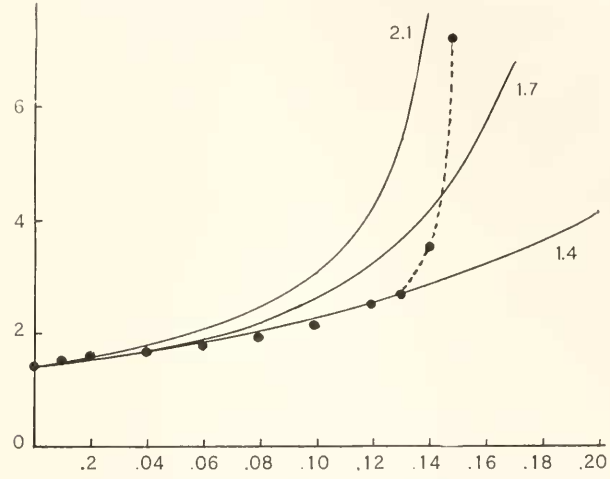

FIG. 1. Observed times for hemolysis of ox erythrocytes in distilled water and in saccharose solutions of different concentrations represented by the solid circles. Calculated times for different values of R represented by the curves. Ordinates represent times; abscissae, concentrations in mols per liter.

IV

A careful examination of the data suggests that the reason for the discrepancy between observation and theory lies not so much in the inapplicability of simple osmotic laws to the hemolytic process as in a serious error in obtaining experimentally the value of the critical hemolytic concentration, c_h. This error, in turn, is responsible for one in the value of R which appears in equations (5) and (6). An inspection of these equations, or even better, the actual substitution in them of several different values of R, all other figures remaining the same, shows that the effect of this constant on the calculated values of k' is very great and that even a small error in its determination must have serious effects. Now it might seem that of all the values entering into the calculations that of c_h, and consequently also that of R, are the most reliable, since the concentration of the solution in which a given degree of hemolysis—for example, 75 per cent—is finally

reached can be determined by direct observation with a very high degree of accuracy. But before using for purposes of calculation values of c_h and of R as so determined account must be taken of a very suspicious circumstance, namely, that the osmotic pressure of the solution of sucrose which ultimately gives 75 per cent hemolysis is very different from those of diluted serum or of NaCl having the same hemolytic effect.

For example, in four separate experiments to be described in a later paper, it was found that the unbuffered solutions of NaCl in which 75 per cent hemolysis was ultimately attained with a dilution of blood of approximately 1 : 500 had concentrations of 0.097M, 0.093M, 0.104M and 0.098M, with an average value of 0.098M. The freezing point depression of such a solution is approximately 0.340°, giving a value of R of 1.7 instead of 2.1 as determined above. In other words, erythrocytes in solutions of saccharose and, as may readily be shown, in solutions of other non-electrolytes also, have a higher resistance than in solutions of electrolytes or in diluted serum. This effect has been noted by many workers and has been variously explained. Leaving undetermined for the present the exact mechanism by which it is produced we may accept it as a known fact and consider some of its possible consequences.

When an erythrocyte is placed in a non-electrolyte solution it possesses certain osmotic properties which are changed by exposure to its new surroundings. The critical hemolytic concentration as actually determined in such solutions is, therefore, obviously that of a cell whose properties have been modified and not that which the unaltered erythrocyte ought theoretically to show. But suppose, as seems likely, that this change is not an instantaneous one, but requires an appreciable time, say, 10 seconds, for its completion. Evidently under these conditions very rapid hemolytic processes requiring only two or three seconds would be finished before much change in the erythrocyte could occur; on the other hand, if the duration of the hemolytic process were 15 or 20 seconds, it would be sufficiently slow to permit the change—in this case an increase in the resistance of the erythrocyte—to take place. Indeed, a point would rather suddenly be reached where the increased resistance developed by the erythrocyte would be sufficient to prevent completely the hemolysis that would otherwise occur; and at this point there would be a sudden rise of the time-of-hemolysis curve to infinity.

As has been noted above, the curve expressing the observed relation between concentration and time of hemolysis shows as its most striking peculiarity a sudden rise as the highest concentrations are

approached. By far the simplest explanation of this peculiarity—indeed the only one that has so far occurred to the author—is that the increased resistance which is known to be brought about in non-electrolyte solutions does not develop instantly but requires for its appearance several seconds—almost certainly more than two or three. If this rather plausible view be accepted, then it is obviously erroneous to use the observed value of c_h in calculations involving water and very dilute solutions in which hemolysis occurs in from 1.4 to 2 or 3 seconds, *i.e.*, before the original osmotic properties of the erythrocyte have been greatly altered. Some other higher value of c_h would evidently govern the behavior of the erythrocyte in such media. Let us assume as a first approximation that the true value of c_h is that determined by the use of NaCl rather than by sugar solutions. From the figures given above, the new value of R would be in the vicinity of 1.7 (*i.e.*, 0.58 ÷ 0.340). Using this figure as preferable to the old one of 2.1 there have been calculated the new values of k' in column 2 of Table II.

TABLE II

Values of k' calculated from the data of Table I, assuming $R = 1.7$ and $R = 1.4$.

Concentration	$Rk' = 1.7$	$Rk' = 1.4$	Concentration	$Rk' = 1.7$	$Rk' = 1.4$
0.00	1.16	0.59	0.08	1.34	0.63
0.01	1.11	0.51	0.10	1.39	0.64
0.02	1.12	0.56	0.12	1.46	0.63
0.04	1.20	0.59	0.13	1.48	0.62
0.06	1.29	0.62	0.14	1.32	0.53

It is immediately apparent on examination of this table (see also the curve labeled 1.7 in Fig. 1) that the agreement between observation and theory, as indicated by the relative constancy of k', is now considerably better than before, though there is still a slow drift in the constant, which can scarcely be accounted for by experimental errors. It is to be noted, however, that the critical concentration as inferred from NaCl experiments is itself probably too low. According to Ponder and Saslow (1931), in experiments whose duration is of the order of magnitude of the time used to determine this figure (1 hour), there is a change, interpreted by them as due to an escape of salts from the erythrocyte, which is sufficient to influence the cell-volume and which would undoubtedly render hemolysis by hypotonic solutions more difficult than otherwise. If this conclusion be accepted, then the observed critical concentration, c_h, is still too low and the assumed value of R of 1.7 is too high.

Though it is impossible at present to be certain what further

correction is justified, it may be of interest to assume for the unaltered cell a value of R of 1.4. Calculations made by using this figure are given in column 3 of Table II. It will be noted that the value of k' is now almost constant, indicating an agreement of theory and observation, up to a concentration of 0.13M. This agreement is even more strikingly shown in Fig. 1 where the curve labeled 1.4 has been calculated for this value of R by means of equations (5) and (6), starting with 1.40 seconds as the time required for hemolysis in distilled water. An even better agreement could be obtained by taking a slightly lower value of R; but, in view of the simplifying assumptions used in deriving the equations, it is questionable whether the almost perfect fit that could be secured in this way has any very great significance. The important thing is that by assigning a not-improbable value to c_h (the theoretical hemolytic concentration for *the unaltered cell*), the behavior of the erythrocyte over a wide range of concentrations shows a good agreement with simple osmotic laws, and its deviation from these laws at very high concentrations can be plausibly accounted for.

V

Up to this point the increased osmotic resistance of the erythrocyte in solutions of non-electrolytes has been accepted merely as an observed fact with no attempt at an explanation. Though for present purposes it is not strictly necessary that the cause of this peculiarity of the erythrocyte should be known, it may be noted that there are not lacking a number of more or less plausible explanations which because of their general theoretical interest may now be briefly considered.

The first explanation is that non-electrolytes actually have a toughening and strengthening effect upon the cell membrane which renders the erythrocyte less susceptible to hemolysis (see for example Rhode, 1923). Although a solidifying effect of non-electrolytes upon certain gels and upon both plant and animal cells has frequently been observed (for literature upon this subject see Höber and Memmesheimer, 1923; and Höber, 1926), it seems very unlikely that this explanation is capable of accounting for such a remarkable increase in the osmotic resistance of the erythrocyte as is known to occur. In the first place, the membrane of this cell is so delicate that it probably offers little opposition under any conditions to volume changes (for some of the evidence see Jacobs, 1931). Even the much better developed membrane of the egg of *Arbacia* seems to be capable of resisting only very feebly osmotic volume changes (Lucké and McCutcheon, 1932; Harvey, 1931; Cole, 1932). It is almost inconceivable that the membrane of the erythrocyte could be so strengthened

in the absence of electrolytes as to support an excess osmotic pressure of over an atmosphere (*i.e.*, the difference in the osmotic pressures of solutions of NaCl and of saccharose, which just permit 75 per cent hemolysis to occur). Furthermore, according to Ponder and Saslow (1931) osmotic hemolysis does not necessarily involve any very appreciable stretching of the cell membrane. It seems unlikely, therefore, that a direct mechanical effect of this sort on the cell is chiefly involved.

A second possibility is somewhat more plausible. It is that in a hypotonic non-electrolyte solution there is a sufficient leakage of electrolytes from the interior of the cell to lower the internal osmotic pressure and so to reduce the amount of swelling that would otherwise occur. This explanation of the increased osmotic resistance of the erythrocyte in non-electrolyte solutions has been accepted, among others, by Bang (1909) and by Ponder and Saslow (1931). The last named authors suppose "that leakage is greater in glucose than in NaCl and that this accounts for the critical volume being reached in a solution of glucose which is more hypotonic than one of NaCl." They cite in support of this view the direct chemical evidence obtained by Kerr (1929) that in solutions deficient in blood proteins there may be an escape of potassium from and an entrance of sodium into the cell. Bang (1909) also gives references to earlier work indicating a passage of electrolytes from the erythrocyte into non-electrolyte solutions, while Joel (1915) has studied this process and the influence upon it of narcotics, by an electrical conductivity method. That electrolytes may escape from the erythrocyte into non-electrolyte solutions may therefore be regarded as a well-established fact.

It is very questionable, however, whether such an escape of electrolytes, which is probably associated with a loss of the normal permeability of the cell to cations, is capable of accounting for the very rapid rise of osmotic resistance that occurs in the present experiments. From the data shown graphically in Fig. 1 it would seem that a marked increase in resistance in non-electrolyte solutions must occur in less than five seconds, while even under the conditions of their experiments, Ponder and Saslow (1931) state that "the equilibrium volumes are attained within a minute and are maintained for hours." Since the diffusion of cations reported by Kerr (1929) is a process that seems to extend over hours, while the rise in conductivity studied by Joel went on gradually and steadily throughout experiments also lasting up to several hours, it would seem that some factor other than an outward leakage of salts (*i.e.*, of both anions and cations) is involved in the case of very rapid changes. The factor that immediately suggests itself is a

new ionic equilibrium of some sort, attained primarily by the movement of anions such as is known to occur readily in normal erythrocytes. How far the results of Ponder and Saslow with electrolyte solutions can be so explained cannot at present be stated with certainty; but, at all events, it seems likely that the extremely rapid increase in the osmotic resistance of erythrocytes that takes place in non-electrolyte solutions is to be accounted for in this way.

This view is supported by the work of Netter (1928), who has pointed out that theoretically ionic exchanges should by no means be absent between erythrocytes and a surrounding isotonic solution of a non-electrolyte, but that anions from the erythrocytes would tend to be exchanged for OH' ions from the aqueous solution in such a way as to make the interior of the cells more alkaline. This principle has been put to practical use by Bruch and Netter (1930) in obtaining various desired relations between external and internal pH values. Now it is known, especially from the work of Warburg (1922) and of Van Slyke, Wu and McLean (1923), that a change in reaction within the erythrocyte has important osmotic consequences. The osmotic pressure of a given amount of base bound by hemoglobin is considerably lower than that of the same amount of base bound by, for example, carbonic acid. If the compound of base with hemoglobin be represented as B_nHb, the osmotic pressure of this compound when completely dissociated would be to that of the same amount of base combined with carbonic acid as $(n + 1) : 2n$. Anything, therefore, which causes a shift of base from hemoglobin to carbonic acid should increase the internal osmotic pressure of the cell and cause the latter to swell; anything that causes a shift in the reverse direction should have the opposite effect. Since the new ionic equilibrium attained in non-electrolyte solutions is obviously of the latter nature, it should, without any escape of salts as such, result in a lowering of the internal osmotic pressure, and so raise the resistance of the cell to hemolysis by hypotonic solutions.

If this view of the mechanism of the increase in the osmotic resistance of the erythrocyte in non-electrolyte solutions be correct, then it ought to be possible even in solutions of electrolytes to produce the same characteristically sudden rise in the time-of-hemolysis: concentration curves described above by slightly increasing the alkalinity of the medium with a trace of NaOH or NH_4OH. This is, in fact, the case, as will be shown in a forthcoming paper by the author in collaboration with A. K. Parpart. For this reason, as well as for the others mentioned above, it seems likely that while a leakage of salts (*i.e.*, of both anions and cations) is by no means excluded as a possible factor of importance in experiments of longer duration, the factor

chiefly concerned in producing increased resistance under the conditions of these experiments is an entirely normal shift of anions alone, which because of certain peculiarities of hemoglobin, is secondarily responsible for a change in the internal osmotic pressure of the cell.

VI

It is a matter of some interest to determine the value of the true permeability constant, k, of equations (1), (3), and (4). This is a measure of the amount of water that would cross the membrane of the erythrocyte through unit area in unit time with unit difference in osmotic pressure between the cell and the surrounding solution. Such a constant would be useful for comparing the permeability to water of different cells, or of the same cell under different conditions (see Jacobs 1927, 1931).

The true permeability constant, k, is found from k' of equations (5) and (6), by multiplying by V_0, the initial osmotically effective volume of the cell, which may be measured in cubic micra, and dividing by A, the area of the cell, which is conveniently expressed in square micra. Unfortunately, both because of the small size of the erythrocyte and its peculiar shape, it is difficult to measure its surface with very great accuracy. This difficulty is well illustrated by the fact that the estimates made by different investigators for the erythrocytes of the same species of mammals may differ by as much as 50 per cent or more. Furthermore, even though the total volume of the cell can be fairly accurately determined by several methods (Ponder and Saslow, 1930) there has in the past been much uncertainty in calculating from it the osmotically effective volume, though it seems likely that this is in reality not very different from that of the total water contained in the cell (Hill, 1930). Finally, there is the serious difficulty, mentioned above, that because of uncertainty as to the value of the theoretical critical hemolytic concentration for the unaltered erythrocyte the value of k' itself is subject to a considerable error. It is evident, therefore, that the most that can be expected at present is to obtain the order of magnitude of the true permeability constant; but even this would be of considerable value.

For the ox erythrocytes chiefly used in these experiments we may take as a first approximation a value of R of 1.7. This value is obtained from the observed hemolytic concentration of NaCl rather than from that of saccharose, since, as has been mentioned above, the normal osmotic properties of the erythrocyte are very quickly changed in non-electrolyte solutions. Indeed, even the value selected is probably somewhat too high, but in the absence of more complete information it is a convenient one to use.

As to the necessary constants for the cell itself, the few published estimates are not in very good agreement. Probably the best available value for the volume of the ox erythrocyte is that given by Ponder and Saslow (1930) of 44 cubic micra. No estimates of surfaces are given in this paper, but in an earlier publication Ponder (1924) has given 37 cubic micra and 69 square micra as the values of the volume and surface, respectively, of the erythrocyte of the calf. If it be assumed that the shape of the somewhat larger cell is exactly the same as that of the smaller one, then its surface would be $69 \times \left(\frac{44}{37}\right)^{2/3}$ or 77 square micra, and this value will here be used.

In the absence of accurate chemical analyses of ox erythrocytes, it may tentatively, though perhaps somewhat questionably, be assumed that they contain the same percentage of water as those of man and that all of this water is "free." Taking an average of the figures given by Henderson (1928) for cc. of water in 1 liter of cells for three normal human individuals, and applying the same percentage to the ox erythrocyte whose total value is 44 cubic micra, we have for the initial effective osmotic volume, V_0, 0.69×44 or approximately 30 cubic micra. Remembering that the time for 75 per cent hemolysis in water is 1.4 seconds, we have all the data necessary to calculate from equation (6) the value of k.

$$k = 0.08 \times \frac{1}{1.4} \times \frac{1}{1.16} \times \frac{30}{77} \times [(1.7)^2 - 1] = 0.036.$$

The factor 0.08 has been introduced to change freezing point depressions in degrees centigrade into osmotic pressures in atmospheres. Expressed in words, the value of k so obtained means that with a difference in osmotic pressure of one atmosphere between the cell and its surroundings water should theoretically pass through each square micron of the cell surface at the rate of 0.042 cubic micra per second or of 2.2 cubic micra per minute.

In an earlier paper (Jacobs, 1927) the value of k was estimated to be of the order of 3.0 for human erythrocytes when the unit of time was taken as one minute. Since the details of the calculation were not given at that time, it may be worth while to present here an additional typical set of figures, emphasizing at the same time the fact that only the general order of magnitude of the results obtained from them is significant. In this particular case c_h in terms of freezing point depressions was found to be 0.232, while c_0 was taken as 0.56, giving a value of R of 2.4. The observed time of hemolysis was 2.4 seconds. Following Emmons (1927) the volume of the human erythrocyte may

be taken as 88 cubic micra (of which 0.69×88 or 61 cubic micra represents the true osmotic volume) and the surface as 145 square micra. We have, therefore:

$$k = 0.08 \times \frac{1}{2.4} \times \frac{1}{1.12} \times \frac{61}{145} \times [(2.4)^2 - 1] = 0.060$$

or, if the unit of time be taken as the minute, 60 times this value or 3.6.

In view of the large unavoidable errors in these calculations, due especially to uncertainty as to the exact value of R, it is questionable whether this apparently greater permeability to water of the human erythrocyte as compared with that of the ox is significant. In any case, the difference in the permeabilities of the two kinds of erythrocytes to water is far less than is that to glycerol (Jacobs, 1927, 1931) or to erythritol and to certain ions (Mond and Gertz, 1929).

Summary

1. Equations are derived for predicting the relation between the time required for osmotic hemolysis and the concentration of a surrounding hypotonic medium.

2. It is shown that when allowance is made for certain known peculiarities of the erythrocyte the rate of hemolysis is, on the whole, in fairly good agreement with osmotic laws.

3. Reasons are given for believing that the increased osmotic resistance of the erythrocyte that develops within a few seconds in solutions of non-electrolytes is not caused by a leakage of salts from the cell but rather by a changed ionic equilibrium in which the normal impermeability of the cell to cations need not be lost.

4. Rough quantitative estimates are made of the permeability of the erythrocytes of the ox and of man to water.

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